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Insulin Degradation by Adipose Tissue

STUDIES AT SEVERAL LEVELS OF CELLULAR ORGANIZATION

Barry J. GOLDSTEIN and James N. LIVINGSTON
Departments of Biochemistry and Medicine, University of Rochester School of Medicine, Rochester, NY 14642, U.S.A.

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A systematic study of the degradation of physiological concentrations of ^{125}I-labelled insulin was performed in intact fat-pads, isolated adipocytes and subcellular fractions of isolated adipocytes. The findings indicate that insulin is rapidly degraded to low-molecular-weight peptides and/or amino acids by the intact tissue and isolated cells. Of the total insulin-degradation products present after incubation with an intact fat-pad, 94% is recovered in the medium, indicating that these products are not retained by the cells or tissue. The plasma membranes do not degrade insulin significantly in the absence of reduced glutathione, and over 99% of the cellular degradative capacity is found in the postmicrosomal supernatant (cytosol). The cytosol degrades insulin to several labelled fragments that are intermediate in size between insulin and insulin A chain, as well as to the low-molecular-weight tissue degradation products. Inclusion of plasma membranes with cytosol accelerates the cleavage of the intermediate fragments to the size of the small products seen with the intact tissue. However, plasma membranes do not increase the initial step in the degradation of insulin when incubated with cytosol, suggesting that the insulin receptor is not involved with the direct cleavage of insulin. This study supports the hypothesis that the bulk of insulin degradation occurs in the adipocyte cytosol, where intermediate-sized fragments are generated and rapidly cleaved to smaller products by the plasma membrane and quickly released into the surrounding medium.

The degradation of insulin is one consequence of the interaction(s) between the hormone and a target cell (Freychet *et al.*, 1972; Gammeltoft & Gliemann, 1973; Terris & Steiner, 1975). Although this process has long been recognized and studied in many different tissues (for a review see Izzo, 1975), only recently have attempts been made to link the events in degradation with those responsible for eliciting hormonal effects. Impetus for seriously considering this possible relationship comes from the demonstrations that insulin is internalized by target cells (Gorden *et al.*, 1978), and that insulin degradation may be related either directly or indirectly to insulin–receptor association (Terris & Steiner, 1975; Gliemann & Sonne, 1978), the initial step in insulin action (Kahn, 1976). Moreover, the persistent failure to identify a second messenger that helps mediate the actions of insulin has also stimulated attempts to show that insulin fragments are intracellular mediators of insulin action (Fujino *et al.*, 1977).

The adipocyte represents a suitable insulin target cell to investigate the process of insulin degradation and its relevance to insulin action. With this cell, it is possible to examine several aspects of insulin action [e.g. association of insulin with the insulin receptor (Gliemann *et al.*, 1975), the stimulation of glucose transport and metabolism, as well as effects on other cellular systems (Minemura *et al.*, 1970; Livingston & Lockwood, 1975; Czech, 1976)], while monitoring the destruction of the hormone. Although insulin degradation by adipose tissue has been previously studied (Rudman *et al.*, 1966; Hammond & Jarett, 1975; Sumner & Doisy, 1970), in general very little is known of the specific physiological events that operate to destroy the hormone. Without this information it is not possible to judge if products of degradation are involved in producing some of the cellular effects of insulin.

In the present work, insulin degradation was studied at physiological hormone concentrations in a

Abbreviations used: V_0, $V_{\text{Ins.}}$, V_A and V_i, the gel-filtration elution volumes for Blue Dextran, insulin, insulin A chain and the column internal volume respectively; GSH, reduced glutathione.

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systematic manner by using intact fat-pads, isolated adipocytes, and subcellular fractions of isolated adipocytes. The results of these studies suggest that insulin degradation is an ordered process, with most of the initial degradation occurring in the cytoplasm. The plasma membranes act to accelerate further degradation of insulin fragments generated in the cytosol to amino acids and/or small peptides, which are rapidly ejected from the cell.

Experimental

Animals

Male Sprague–Dawley rats weighing 220–240g were obtained from Charles River Laboratories.

Materials

$Na^{125}I$ was purchased from New England Nuclear. Porcine insulin was a gift from the Eli Lilly Co. Bio-Gel P-10 (100–200 mesh) was obtained from Bio-Rad Laboratories. Oxidized insulin A chain, cytochrome *c* (type VI), NADH, ATP, Ficoll (type 400DL) and Blue Dextran were purchased from Sigma Chemical Co. Anti-[guinea-pig immunoglobulin G (goat)] and anti-[bovine insulin (guinea pig)] sera were obtained from Miles Laboratories. Dialysis tubing (type H47) and silicone oil were purchased from Arthur H. Thomas Co., Philadelphia, PA, U.S.A. Collagenase (type I) was supplied by Worthington, and bovine serum albumin (fraction V) was obtained from Reheis Chemical, Phoenix, AZ, U.S.A. Sucrose (ultrapure) was a product from Schwarz/Mann. All other materials used were of reagent grade.

Methods

Preparation of ^{125}I-labelled insulin. ^{125}I-labelled insulin was prepared by the chloramine-T method to a specific radioactivity of 100–200Ci/g and purified on talc (Cuatrecasas & Hollenberg, 1976). The hormone was further purified immediately before use by gel filtration on a column (1cm × 25cm) of Bio-Gel P-10 in Krebs–Ringer phosphate buffer (Livingston & Lockwood, 1975) prepared without calcium or magnesium and containing 0.1% (w/v) bovine serum albumin. Approx. 91% of the radioactivity was located on the A chain of insulin, as determined by isoelectric focusing after sulphitolysis (Antoniades *et al.*, 1974) of the radiolabelled hormone. Rechromatography of the purified ^{125}I-labelled insulin gave a single peak of radioactivity eluted from the Bio-Gel P-10 column (1.6cm × 100cm) described below (results not shown). Radioactivity was determined with a Searle model 1197 gamma counter.

Degradation of ^{125}I-labelled insulin by intact fat-pads, isolated adipocytes and subcellular fractions. Rats were allowed free access to food before being killed by a sharp blow to the head. Two intact epididymal fat-pads were removed by single cuts of their proximal ends to avoid cellular damage, and the distal heads were suspended in 4ml of buffer A (Krebs–Ringer phosphate buffer, pH7.4, containing 30mg of bovine serum albumin/ml), containing ^{125}I-labelled insulin (0.5nM), and incubated in an atmosphere of O_2/CO_2 (19:1) at 37°C for 90min. The fat-pads were quickly rinsed with 5ml of buffer at room temperature, and rapidly homogenized in 0.8ml of buffer B [4M-urea/1M-acetic acid/0.1% (v/v) Triton X-100] (Kahn & Baird, 1978) with a Dounce homogenizer after being minced with scissors. The homogenate was centrifuged at 29000*g* for 30min at 4°C. A sample of the supernatant material was then analysed by gel filtration.

Isolated adipocytes were prepared by the collagenase method from the epididymal fat-pads of 12 rats as previously described (Rodbell, 1964). The cells were washed gently three times with several volumes of buffer A and diluted 1:3 in buffer A. Then 5ml of this suspension was incubated with 0.5nM-^{125}I-labelled insulin under an atmosphere of O_2/CO_2 (19:1) at 37°C for 30min. The cells were centrifuged through 2ml of silicone oil at 1000*g* for 5min at room temperature. The cell pellet was dissolved in 1ml of buffer B and treated as above for the fat-pad. A sample of the supernatant material from the cells as well as a portion of the incubation medium was then analysed by gel filtration.

Alternatively, adipocytes were further washed twice with several volumes of buffer C [10mM-Tris/HCl (pH7.4)/1mM-EDTA/0.25M-sucrose] (Jarett, 1974) after isolation for the preparation of subcellular fractions. The fractions were prepared exactly as described by Jarett (1974) by using differential centrifugation and a Ficoll/sucrose discontinuous gradient to purify plasma membranes. The postmicrosomal supernatant is described in the present report as cytosol. The nuclear and mitochondrial adipocyte fractions were discarded, since they have previously been shown to contain insignificant amounts of insulin-degrading activity (Hammond & Jarett, 1975). All cellular materials were stored at 4°C and used within 48h of preparation.

Adipocyte subcellular fractions were incubated with 0.5nM-^{125}I-labelled insulin in buffer A or buffer D (5mM-EDTA/10mM-Tris-HCl, pH7.4) containing 30mg of bovine serum albumin/ml at 37°C in a final volume of 0.15 or 0.4ml. All procedures were performed with plastic labware. At the appropriate times, samples were removed for assay of insulin degradation (see below) or, alternatively, 1vol. of 2 × buffer B (i.e. double concentrations) was added to stop the reactions and prepare the sample for column chromatography.

Characterization of subcellular fractions. Immediately after preparation, subcellular fractions

were assayed for marker enzyme activities. ($Na^{+}+K^{+}+Mg^{2+}$)-stimulated ATPase was measured as described by McKeel & Jarett (1970), and P_i was quantified by the method of Hurst (1964). NADH-dependent cytochrome *c* reductase was assayed by the method of Dallner *et al.* (1966). Protein was measured as described by Miller (1959).

Trichloroacetic acid-solubility assay for insulin degradation. Degradation of ^{125}I-labelled insulin was measured by an increase in trichloroacetic acid solubility of radioactivity in the incubation mixture. A 50μl sample was removed and added to 300μl of 12% (w/v) trichloroacetic acid at the required time. Precipitation was allowed to occur at a final bovine serum albumin concentration of 4mg/ml and 10% (w/v) trichloroacetic acid, as suggested by Gammeltoft & Gliemann (1973), for 10min at 4°C. The suspension was then transferred to a small plastic tube and centrifuged at 10000*g* for 3min in a Microfuge (Beckman). A portion of the supernatant radioactivity was counted and compared with a portion of the initial incubation mixture to calculate the percentage trichloroacetic acid-soluble radioactivity.

Immunoprecipitation assay for insulin degradation. ^{125}I-labelled-insulin degradation was quantified alternatively by measuring a loss of substrate immunoreactivity as described by Lomedico & Saunders (1976), except that 3mM-*N*-ethylmaleimide was included in the immunoprecipitation buffers. The purified ^{125}I-labelled insulin preparation was 100% immunoprecipitable by this method and over 97% of the precipitated radioactivity was displaced by excess (10μM) unlabelled insulin.

Gel filtration of insulin-degradation products. Column chromatography was performed by applying 0.3–0.6ml samples from the incubation mixtures in buffer B to a column (1.6cm × 100cm) of Bio-Gel P-10, and the column developed with buffer B at room temperature. Fractions of 1.65 or 1.8ml were collected with upward-flow elution at a rate of 10ml/h, and the radioactivity in each fraction was determined. Standardization of the column was performed by using Blue Dextran (V_0), ^{125}I-labelled insulin ($V_{ins.}$), ^{125}I-labelled oxidized insulin A chain (iodinated by the chloramine-T method) (V_A) and $Na^{125}I$ (V_i).

Results

Degradation of ^{125}I-labelled insulin by the intact fat-pad

Studies of insulin degradation were initially performed in intact fat-pads to circumvent possible artifacts arising from the collagenase treatment used in the preparation of isolated adipocytes, a cell system generally used in studies of insulin action and insulin degradation (Gliemann & Sonne, 1978; Kahn & Baird, 1978). In these experiments, the uncut portions of the fat-pads were carefully suspended in buffer A to reduce the release of intracellular degrading activity. After a 90min incubation of the fat-pads at 37°C with ^{125}I-labelled insulin (0.5nM), 9% of the hormone was destroyed, as measured by trichloroacetic acid solubility. Of the trichloroacetic acid-soluble products, approx. 94% were present in the incubation medium and 6% were found in the fat-pads. These results have been corrected for the insulin-degrading activity released into the incubation medium (Gliemann & Sonne, 1978) in order to reflect only tissue-derived degradation products. These findings indicate that the intact fat-pad degrades insulin and that the products of degradation rapidly exit the tissue.

Fig. 1 shows a Bio-Gel P-10 gel-filtration pattern for the ^{125}I-labelled material present in the fat-pads. Four peaks of radioactivity are evident: a void-volumn peak (V_0), an insulin-containing peak ($V_{ins.}$) and two peaks of radioactivity at the internal column volume (V_i). It should be noted that primarily labelled A chain fragments are observed in these experiments, since the ^{125}I-labelled insulin is prepared with more than 90% of the radioactivity on the A chain (see under 'Methods'). The radioactivity eluted in the V_i region appears as two incompletely resolved peaks and suggests small-product heterogeneity. The elution profile for the radioactivity in the incubation medium has the same major peaks, including the two peaks in the V_i region (results not shown).

A time-dependent accumulation of labelled material in the V_0 peak is consistently observed after

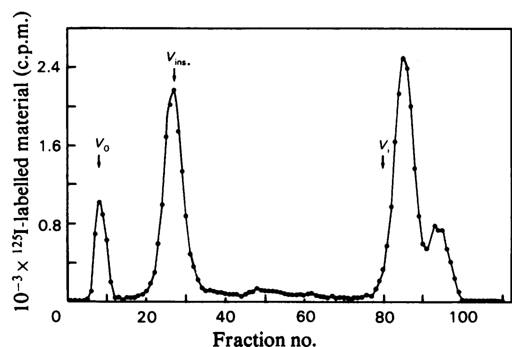

Fig. 1. *Gel-filtration profile of ^{125}I-labelled-insulin-degradation products associated with intact fat-pads*
Two epididymal fat-pads were incubated with purified 0.5nM-^{125}I-labelled insulin and homogenized as described in the text. After centrifugation, a sample of the supernatant material in buffer B was subjected to gel filtration on a Bio-gel P-10 column (1.6cm × 100cm) eluted with buffer B. Fractions (1.65ml) were collected at a flow rate of 10ml/h, and the radioactivity in each fraction was determined.

incubation of ^{125}I-labelled insulin (0.5nM) at 37°C in buffer A in the absence of cellular degrading activity. Under these conditions, no label appears to be eluted after the insulin-containing peak; however, up to 16% of the applied radioactivity may appear in the V_0 after a 60min incubation at 37°C.

Studies with isolated adipocytes

The heterogeneity of tissue components present in an intact fat-pad (Napolitano, 1965) prompted the investigation of insulin degradation by isolated adipocytes. A supernatant was prepared from homogenized adipocytes after incubation with ^{125}I-labelled insulin, and the degradation products were analysed by gel filtration exactly as described for the fat-pad. As shown in Fig. 2(*a*), the major peaks of radioactivity are eluted in positions identical with those in the profile from the intact fat-pad (Fig. 1); this result was previously noted for isolated adipocytes (Gliemann & Sonne, 1978; Kahn & Baird, 1978). In contrast, the gel-filtration profile of the products recovered from the incubation medium (Fig. 2*b*) demonstrates the presence of several minor peaks not observed in the profiles from the fat-pad and which occur in only trace quantities in the profile for the isolated cells. Further investigation disclosed that the crude bacterial collagenase used to disperse the cells contains substantial degrading activity for ^{125}I-labelled insulin (Fig. 2*c*). Concentrations of collagenase as low as 2μg/ml render 15% of the ^{125}I-labelled insulin (0.5nM) soluble in trichloroacetic acid after a 2h treatment at 37°C. This activity is dependent on enzyme concentration and is not inhibited by 1mM-*N*-ethylmaleimide (results not shown), which blocks the insulin-degrading activity of adipose tissue (see Table 2). The intermediate peaks observed in the adipocyte medium (Fig. 2*b*) probably originate from contaminating collagenase, since they are co-eluted with labelled fragments generated by collagenase alone (Fig. 2*c*). Furthermore, medium removed from isolated adipocytes incubated in buffer A demonstrates significant insulin-degrading activity and a gel-filtration profile remarkably similar to that obtained with collagenase (results not shown). Substantial contamination due to collagenase components adhering to the isolated cells has been reported, even after thorough washing (Salans & Dougherty, 1971). Because of the presence of this contaminating activity, it was impossible to assess the rate of appearance of ^{125}I-labelled-insulin-degradation products in the medium from the isolated adipocytes.

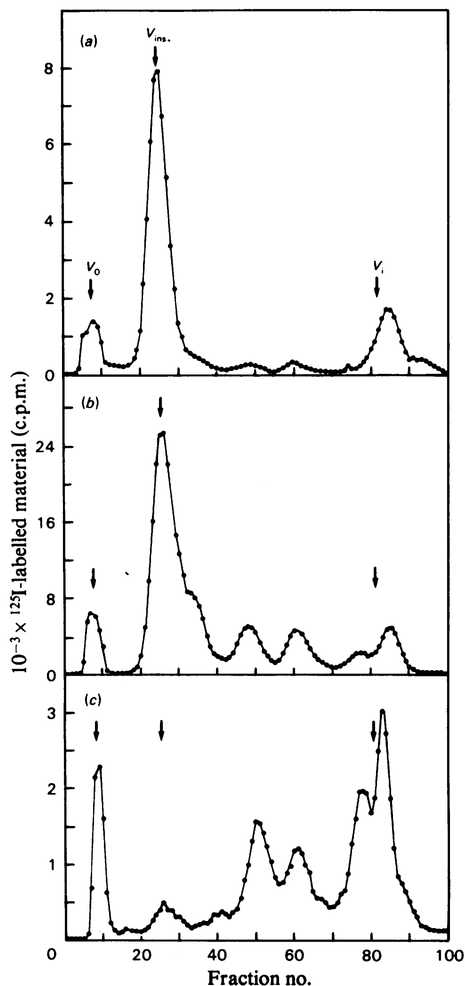

Fig. 2. *Gel-filtration profiles of ^{125}I-labelled-insulin-degradation products*

(*a*) Degradation products associated with isolated adipocytes. Isolated adipocytes prepared by the collagenase method were incubated with 0.5nM-^{125}I-labelled insulin in buffer A and then homogenized in buffer B as described in the text. A portion of the centrifuged homogenate was then subjected to gel filtration as described in Fig. 1. (*b*) The same protocol was used as in (*a*), except that a portion of the medium from the adipocyte incubation with ^{125}I-labelled insulin was diluted 1:2 in 2×buffer B and then subjected to gel filtration as described in Fig. 1. (*c*) ^{125}I-labelled-insulin degradation by crude bacterial collagenase. ^{125}I-labelled insulin (0.5nM) was incubated for 2h at 37°C with 20μg of crude collagenase in 1ml of buffer A. The incubation mixture was then diluted as described in (*b*), and subjected to gel filtration as described in Fig. 1.

Studies with adipocyte subcellular fractions

Insulin degradation was further studied in subcellular fractions of isolated adipocytes prepared after the isolated cells had been washed several times with the homogenization buffer, buffer C. Results for the recovery of protein and the two marker enzymes for the various subcellular fractions are shown in Table 1. The values for protein recovery closely

Table 1. *Distribution of marker-enzyme activities and protein for adipocyte subcellular fractions*
Adipocytes were isolated from the epididymal adipose tissue of 12 rats weighing 220–240g, and subcellular fractions were prepared and used immediately to assay marker-enzyme activities as described in the text. Values represent means ± S.E.M. for three separate experiments. ATPase activity is expressed as nmol of P_i released/10min per mg of protein; reductase activity is given as nmol of cytochrome *c* reduced/min per mg of protein.

Fraction	Total protein (mg)	Enzyme activity	
		$(Na^+ + K^+ + Mg^{2+})$-ATPase	NADH–cytochrome *c* reductase
Homogenate	83 ± 3	140 ± 14	600 ± 40
Cytosol	43 ± 2	13 ± 5	12 ± 3
Plasma membranes	1.0 ± 0.2	650 ± 200	760 ± 90
Microsomal	2.0 ± 0.2	130 ± 5	3100 ± 150

Table 2. *^{125}I-labelled-insulin degradation in adipocyte subcellular fractions*
Adipocyte subcellular fractions were prepared as described in the text and used at the protein concentration indicated in the assay for insulin degradation. ^{125}I-labelled-insulin degradation is expressed as the percentage of the radioactivity of the labelled hormone (0.5nM) soluble in trichloroacetic acid (10%, w/v) after a 30min incubation in buffer A at 37°C; results are given as means ± S.E.M. for the numbers of experiments in parentheses. Values for the total insulin-degrading activity of the fraction are calculated from the degradation rates given in this Table and the protein determinations for each fraction presented in Table 1. Data are given as pmol of insulin degraded after 30min at 37°C measured by the trichloroacetic acid-solubility method, and the values represent means ± S.E.M., incorporating the errors for each separate calculation.

Fraction	^{125}I-labelled-insulin degradation	Total activity
Homogenate (200μg/ml)	10.8 ± 1.0 (4)	23.2 ± 3.0
Cytosol (200μg/ml)	24.9 ± 2.8 (4)	26.8 ± 4.3
Plasma membranes (100μg/ml)	0.7 ± 0.1 (4)	0.035 ± 0.012
Microsomal (100μg/ml)	2.2 ± 0.4 (3)	0.220 ± 0.062
Cytosol (200μg/ml) + *N*-ethylmaleimide (1mM)	0.2 ± 0.1 (4)	—
Cytosol (200μg/ml) + plasma membranes (100μg/ml)	36.8 ± 3.7 (4)*	—

* $P < 0.01$ by paired *t* analysis versus the sum of the cytosol and plasma-membrane incubations performed separately.

agree with those reported by Jarett (1974). The enrichment of plasma membranes for $(Na^+ + K^+ + Mg^{2+})$-stimulated ATPase activity is approx. 5-fold over both the homogenate and the microsomal fraction. Similarly, NADH–cytochrome *c* reductase activity is enriched approx. 5- and 4-fold in the microsomal fraction over the homogenate and the plasma-membrane fraction respectively. These values are in close agreement with those reported for similar fractions prepared by linear (McKeel & Jarett, 1970) or discontinuous (Jarett, 1974) Ficoll/sucrose gradients.

Among the three fractions studied, more than 99% of the total ^{125}I-labelled-insulin-degrading activity, as measured by the trichloroacetic acid-solubility method, is present in cytosol (Table 2). The insulin-degrading activity present in the cytosol fraction is completely inhibited by 1mM-*N*-ethylmaleimide (Table 2) and therefore cannot be attributed to *N*-ethylmaleimide-resistant proteinases from the collagenase treatment. The specific radioactivity of ^{125}I-labelled insulin degradation (percentage degraded/mg of protein) is 5.7 times higher in the cytosol than in the microsomal fraction, which agrees with the value reported previously for this tissue (Hammond & Jarett, 1975). However, very little degrading activity is found in the plasma-membrane fraction (Table 2).

Purified plasma membranes of adipocytes are known to contain the bulk of cellular insulin-binding activity (Hammond & Jarett, 1975). To assess the effects of the insulin receptor on insulin degradation, plasma membranes were added to cytosol and insulin degradation was assayed by the trichloroacetic acid-solubility method. As shown in Table 2, the presence of plasma membranes increases cytosol insulin degradation by 45%. Under these conditions, insulin degradation is linear for at least 30min. This potentiation of cytosol insulin degradation is dependent on the amount of plasma membranes present; the effect is saturated when 200μg of membrane protein is added to 200μg of cytosol in a 1ml assay (results not shown).

Degradation of ^{125}I-labelled insulin by the plasma-membrane and cytosol fractions was further studied by gel filtration of the degradation products on Bio-Gel P-10 (Fig. 3). In agreement with the previous studies in which degradation was measured by tri-

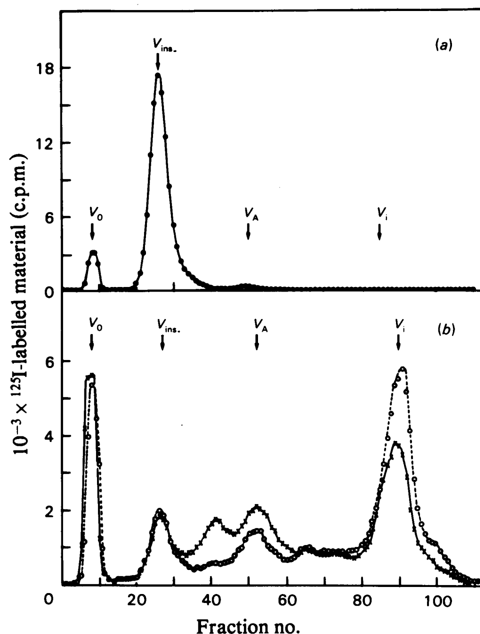

Fig. 3. *Gel-filtration profiles of ^{125}I-labelled-insulin-degradation products from adipocyte subcellular fractions*
(*a*) Plasma membranes (35 μg of protein) were incubated at 37°C for 30 min with ^{125}I-labelled insulin (0.5 nM) in 0.5 ml of buffer A. Then 1 vol. of 2 × buffer B was added, and a sample of this material was analysed by gel filtration as described in Fig. 1. (*b*) The same protocol was used as in (*a*) except that the incubations contained either 100 μg of cytosol protein (×) or 100 μg of cytosol protein with 50 μg of membrane protein (O) in a 0.5 ml volume; the same amount of radioactivity was applied to the column for both experiments; therefore areas under the peaks are representative of the percentage of total radioactivity eluted in that position for each experiment.

chloroacetic acid solubility, the plasma membranes alone are found to destroy only a trace amount of labelled insulin. In contrast, cytosol alone causes extensive destruction of ^{125}I-labelled insulin. Besides the degradation products eluted in the V_0 and V_i, the cytosol produces fragments with peaks of radioactivity eluted in the position of the A chain and in the region between the insulin and the A-chain peaks. These two components do not appear in the elution profiles from the intact adipocytes or fat-pad (Figs. 1 and 2).

Although an increase in trichloroacetic acid-soluble products is observed by incubating plasma membranes with cytosol, this effect is not caused by an increase in the destruction of intact insulin. As shown in Fig. 3(*b*), the amount of radioactivity in the insulin peak is the same in the presence or absence of membranes; also, the size of the V_0 peak is comparable with or without plasma membranes. Therefore the effect of plasma membranes is to accelerate the degradation of the intermediate peak (eluted between $V_{ins.}$ and V_A), which leads to an increase in the amount of low-molecular-weight (V_i) products.

Table 3. *^{125}I-labelled-insulin degradtion by plasma membranes in the presence of 2 mM-GSH*
Incubations were performed at 37°C for 90 min in buffer D containing 0.5 nM-^{125}I-labelled insulin and 30 mg of bovine serum albumin/ml. A sample was analysed by gel filtration as described in Fig. 3. Plasma membranes were incubated at a protein concentration of 160 μg/ml.

Incubation	Percentage of total radioactivity in each peak		
	V_0	$V_{ins.}$	V_A
Buffer	23	50	27
Plasma membranes	15	19	66

To investigate the possibility that the adipocyte plasma membrane contains an enzyme with glutathione–insulin transhydrogenase activity as reported for liver (Varandani, 1973), plasma membranes were incubated with ^{125}I-labelled insulin and a physiological concentration of GSH (2 mM) (Goldstein & Livingston, 1978). As indicated in Table 3 and as previously described (Hird, 1962), GSH itself has significant activity in reducing insulin to labelled material eluted in the V_A region of the gel-filtration column. However, plasma membranes greatly accelerate this reductive cleavage (Table 3), but under these conditions, no further degradation products of the A chain are observed (results not shown).

The degradation of ^{125}I-labelled insulin by the microsomal fraction produces labelled material that is eluted in the position of the A chain and a smaller peak that appears shortly after the A chain (Fig. 4). Microsomal fractions are therefore similar to cytosol, since they digest insulin to intermediates that are not observed in the intact tissue. It is important to note that degradation products do not appear in the internal column volume, which is similar to the results obtained with plasma membranes. However, the substantial amount of degradation products located in the V_A position clearly differentiates the degradative activity of the microsomal fraction from that of plasma membranes.

Results with dialysed cytosol

The effect of dialysis on the insulin-degrading activity of adipocyte cytosol was studied to determine if this activity is dependent on a diffusible factor such as GSH. Preparation of the dialysis tubing by the usual EDTA treatment leads to a significant loss of degrading activity, as assessed by

Table 4. *Effect of dialysis and GSH on ^{125}I-labelled-insulin degradation*
Incubations were performed at 37°C for 30min in buffer A with 0.5nM-^{125}I-labelled insulin and cytosol protein at 200μg/ml (trichloroacetic acid-solubility assay) and 100μg/ml (immunoprecipitation assay); where included, membrane protein was 160 and 80μg/ml for the two assays respectively. Values are corrected for backgrounds in the absence and presence of GSH (2mM), and represent the means of two experiments. Dialysis was carried out with 2ml of the cytosol from the fractionation procedure against 1 litre of buffer C at 4°C for 24h with two changes. The dialysis tubing was treated, to remove sulphur compounds, with 0.3% Na_2S at 80°C for 1min, and washed with water and 0.2% H_2SO_4 before equilibration with buffer C.

Incubation	^{125}I-labelled insulin degraded (%) Trichloroacetic acid-solubility assay	Immunoprecipitation
Cytosol	16.2	28
Cytosol + GSH	10.8	35
Dialysed cytosol	15.5	35
Dialysed cytosol + GSH	8.8	36
Plasma membranes	1.2	7.5
Cytosol + plasma membranes	27.9	35
Dialysed cytosol + plasma membranes	29.3	39

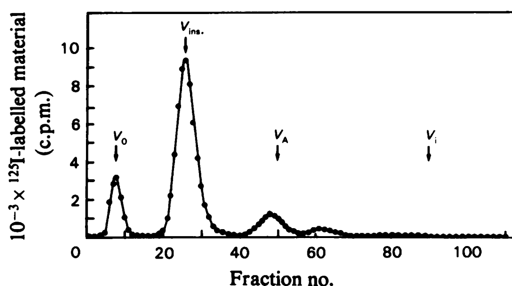

Fig. 4. *Gel-filtration profile of ^{125}I-labelled-insulin-degradation products from adipocyte microsomal fraction*
Adipocyte microsomal fraction (66μg of protein) was incubated for 60min at 37°C with ^{125}I-labelled insulin (0.5nM) in 0.4ml of buffer A. A sample was prepared and gel filtration was performed as described in Fig. 3(*a*).

either method (results not shown). However, when the tubing is treated with sulphide to remove sulphur compounds, no such loss of activity occurs (Table 4). These results suggest that the cytosol enzyme(s) is not dependent on a diffusible factor for activity, and that the enzyme(s) is sensitive to sulphurous contaminants on the dialysis tubing.

The effects of GSH on the degradation of insulin by cytosol are complex and difficult to interpret. There is clearly a difference in the results depending on which method is used to assess insulin destruction. For example, the addition of GSH to the undialysed cytosol inhibits the formation of trichloroacetic acid-soluble products, but potentiates insulin destruction when measured by immunoprecipitation (Table 4). Dialysed cytosol responds to GSH treatment in a similar manner to the undialysed material, suggesting that GSH is not an important participant in the degradation of insulin by this cell compartment.

The immunoprecipitation method for assessing insulin degradation also shows that the addition of plasma membranes to cytosol does not potentiate the destruction of intact insulin (Table 4). Since immunoprecipitation measures the amount of intact insulin remaining, this observation confirms the data in Fig. 3(*b*), i.e. plasma membranes do not potentiate the degradation of native insulin, only those fragments of ^{125}I-labelled insulin first produced in the cytosol.

Discussion

The cellular degradation of circulating proteins including polypeptide hormones is a complex and poorly understood phenomenon (Bohley *et al.*, 1970; Ballard, 1977). This process may in some cases be receptor-initiated (Neville & Chang, 1978), and movement to specific intracellular degrading sites may occur (Katunuma *et al.*, 1975). However, although insulin can move into target cells after interacting with the receptor (Schlessinger *et al.*, 1978), it is not clear whether the degradation of insulin follows a designated pathway or whether a variety of proteinases act randomly to destroy the hormone.

There is also difficulty in interpreting the findings from previous insulin-degradation studies. Differences in the methods used to quantify the amount of degradation, the conditions under which the studies were conducted (buffer composition, non-physiological hormone concentrations etc.) and the type of cell preparation (whole tissue, isolated cell and subcellular fraction) all can contribute to produce artifacts and contradictory findings. To help alleviate these problems, insulin degradation by

adipocytes was systematically studied in the present investigation at the organ level (i.e. fat-pad), and in isolated adipocytes and subcellular fractions, with the use of physiological hormone concentrations and three different methods to assess hormonal destruction.

The intact fat-pad degrades insulin and produces products of two sizes, one of which is eluted in the void volume of the Bio-Gel P-10 column. The other products are small and are eluted as two overlapping peaks in the internal volume. Only these same products are found in the incubation buffer, which contains the vast majority of degraded material. The V_0 material is observed in the absence of cellular material and therefore may not be physiologically significant (see below). Insulin fragments the size of the A chain are not observed; if they occur, their half-life in the tissue must be extremely short. Also, the studies on the fat-pad indicate that the tissue does not accumulate the products of degradation, which appear to be rapidly ejected from the cells. This has previously been suggested for isolated adipocytes (Gliemann & Sonne, 1978). Insulin degradation by this cell preparation resulted in the same products as were found with the fat-pad. In addition, other insulin fragments were found in the incubation medium; however, these products appeared to result from the action of proteinases that contaminate the cell preparation as a consequence of the treatment with crude collagenase (Salans & Dougherty, 1971). Thus no cell-derived products of intermediate size were found, which again suggests the presence of a highly efficient mechanism responsible for insulin degradation.

To investigate the cellular mechanisms comprising this efficient degrading system, subcellular fractions prepared from isolated adipocytes were examined to determine their abilities to degrade the hormone. In the absence of GSH, almost all the degrading activity in the crude homogenate is present in the cytosol fraction (99%; Table 2). The microsomal fraction destroyed insulin at a much higher rate than the plasma membranes, although the microsomal activity was very small compared with that of the cytosol fraction. In the presence of GSH, the membrane fraction was able to degrade more insulin. However, considering the quantity of total activity recovered in the cytosol fraction, membrane degradation is overall a small percentage of the cellular degrading capacity. GSH does not stimulate degradation by the adipocyte cytosol, in agreement with previous findings (Hammond & Jarett, 1975).

Gel-filtration studies demonstrated that the cytosol and microsomal fractions generate fragments of insulin, which are not observed in the fat-pad or isolated adipocytes. One of the fragments is eluted in the position of insulin A chain and the other is eluted between the A chain and insulin. These 'new' intermediate-sized insulin fragments occur as initial degradation products in the adipocyte cytosol. In the intact cell or tissue, these fragments are not observed, suggesting that they have a transient intracellular existence.

A possible explanation for the short half-life of the intermediate-sized insulin fragments generated in cytosol is provided by the effects of plasma membranes on the cytosol degrading ability. Although membranes did not appear to contribute significantly to the initial degradation of the hormone, they did enhance the ability of the cytosol to degrade the intermediate-sized fragments to the small products found in the internal volume (Fig. 3). This enhancement was also shown by the increase in trichloroacetic acid-soluble fragments produced by the combination of plasma membranes with cytosol. In these experiments, the amounts of labelled insulin fragments eluted at the internal column volume correlated directly with the trichloroacetic acid-soluble radioactivity (results not shown). The plasma membranes were also unable to increase the appearance of trichloroacetic acid-soluble products in cytosol when the cytosol degrading activity was dialysed in EDTA-treated dialysis tubing, a manoeuvre that destroys the insulin-degrading ability of cytosol (results not shown). Therefore it is possible that the plasma membrane acts on a fragment of insulin that is slightly larger than the insulin A chain, which is generated in the cytosol; this action further degrades it to the products eluted at the internal column volume. These findings suggest the existence of a co-operative mechanism between the plasma membrane and cytosol for the rapid and highly efficient degradation of insulin to small products. The location of this activity on the plasma membrane may help explain the rapid release of these small products from the cell.

The biological relevance of these degradation products is not known. It has been observed that synthetic peptides with homology to sequences on the insulin B chain possess insulin-like activity (Fujino *et al.*, 1977; Weitzel *et al.*, 1971, 1973). Furthermore, one such fragment has been shown to potentiate the actions of insulin in adipose tissue (Fujino *et al.*, 1977). Kahn & Baird (1978) have proposed that the high-molecular-weight material eluted in the void volume may represent a biologically important association of insulin with the insulin receptor or with other cellular components. However, we observe a time-dependent accumulation of this product in the absence of any cellular material, suggesting that it is not physiologically related to insulin metabolism in adipocytes. Although adipocytes and subcellular fractions caused a marked increase in the void-volume material, this may simply represent covalent com-

plexes formed by thiol exchange as the hormone is reductively cleaved (Mahbouba & Smith, 1977). We find that after sulphitolysis most of the radioactivity from this peak migrates as *S*-sulphonated A chain during isoelectric focusing (results not shown), indicating that it may consist of intact insulin chains linked as disulphides in a high-molecular-weight aggregate (Varandani *et al.*, 1972; Antoniades *et al.*, 1974).

The results of the present study suggest that adipocyte subcellular compartments may act in sequence to cleave insulin. Although some degradation of the hormone may occur at the plasma membrane, it appears to enter the cell or in some manner gain access to cytoplasmic enzymes, which produce the bulk of hormone degradation. In fact, the cytosol is the only subcellular adipocyte fraction capable of degrading insulin to the low-molecular-weight material that is observed as the major product of insulin degradation in the intact tissue. The movement of insulin to an intracellular compartment in adipocytes may involve the insulin receptor or the formation of pinocytotic vesicles at the surface of the cell (Barrnett & Ball, 1960). However, the receptors or other plasma-membrane structures do not appear to be involved with the direct degradation of insulin, since the addition of membranes to cytosol does not increase the initial destruction of insulin. Also, it has been demonstrated in our laboratory that the insulin-degrading activity of liver membranes can be separated from the binding activity of the insulin receptor (Livingston & Krupp, 1978). Furthermore, experiments with adipocyte plasma membranes demonstrate that detergent-solubilized material containing the insulin receptor does not degrade insulin (Krupp & Livingston, 1978). It seems clear therefore that, in the adipocyte, insulin destruction is carried out to a large extent by cytoplasmic factors.

The plasma membrane appears to participate in the further processing of the initial (intermediate-sized) fragments of insulin that are generated in the adipocyte cytosol. This co-operation enhances that ability of the recombined subcellular fractions to degrade insulin further to small peptides and/or amino acids, which are observed as the only degradation products in the intact tissue. Some evidence of synergism for the production of trichloroacetic acid-soluble products from insulin has also been observed between a high-speed supernatant and a particulate fraction from the rat kidney (Duckworth, 1976).

In conclusion, insulin degradation by adipocytes may occur as an ordered process whereby fragments produced by one subcellular fraction are subsequently degraded further by another cellular compartment. The low-molecular-weight products are then rapidly released into the surrounding medium and are not accumulated by the cell. These results offer some insight into the cellular processes acting to degrade insulin. It is also possible that similar mechanisms are involved in the intracellular degradation of other circulating proteins.

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